



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> <b>C12N 15/00, 9/64, A61K 37/54</b> <b>C12N 1/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 87/07907</b> <b>(43) International Publication Date:</b> <b>30 December 1987 (30.12.87)</b>
<b>(21) International Application Number:</b> PCT/GB87/00420 <b>(22) International Filing Date:</b> 16 June 1987 (16.06.87) <b>(31) Priority Application Numbers:</b> 8614722 8619272 <b>(32) Priority Dates:</b> 17 June 1986 (17.06.86) 7 August 1986 (07.08.86) <b>(33) Priority Country:</b> GB <b>(71) Applicant (for all designated States except US):</b> CELL- TECH LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> DOCHERTY, An- drew, James, Penrose [GB/GB]; 34 Seymour Road, Hampton Hill, Middlesex TW12 1DD (GB). MUR- PHY, Gillian [GB/GB]; 204 Cambridge Road, Great Shelford, Cambridge CB2 5UV (GB).		<b>(74) Agent:</b> VOTIER, Sidney, David; Carpmaels & Rans- ford, 43 Bloomsbury Square, London WC1A 2RA (GB). <b>(81) Designated States:</b> AT (European patent), BE (Euro- pean patent), CH (European patent), DE (European patent), FR (European patent), GB (European pa- tent), IT (European patent), JP, LU (European pa- tent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt</i> <i>of amendments.</i>
<b>(54) Title: PROCESS FOR THE PRODUCTION OF A PROTEIN</b>		
<b>(57) Abstract</b>		
<p>Process for the production of a mam-  malian stromelysin by a recombinant DNA  process. The invention also relates to specific  proteins, DNA sequences, vectors, host organ-  isms, pharmaceutical compositions, DNA  probes, antibodies and to the use of stromely-  sin to enhance the activity of collagenase.  Stromelysin may be useful therapeutically for  example in the debridement of dermal ulcers,  modification of scar tissue formation arising  from healing of wounds and in the treatment  of herniated vertebral discs.</p> <div style="text-align: center;"> </div> <p>1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97  98  99  100  101  102  103  104  105  106  107  108  109  110  111  112  113  114  115  116  117  118  119  120  121  122  123  124  125  126  127  128  129  130  131  132  133  134  135  136  137  138  139  140  141  142  143  144  145  146  147  148  149  150  151  152  153  154  155  156  157  158  159  160  161  162  163  164  165  166  167  168  169  170  171  172  173  174  175  176  177  178  179  180  181  182  183  184  185  186  187  188  189  190  191  192  193  194  195  196  197  198  199  200  201  202  203  204  205  206  207  208  209  210  211  212  213  214  215  216  217  218  219  220  221  222  223  224  225  226  227  228  229  230  231  232  233  234  235  236  237  238  239  240  241  242  243  244  245  246  247  248  249  250  251  252  253  254  255  256  257  258  259  260  261  262  263  264  265  266  267  268  269  270  271  272  273  274  275  276  277  278  279  280  281  282  283  284  285  286  287  288  289  290  291  292  293  294  295  296  297  298  299  300  301  302  303  304  305  306  307  308  309  310  311  312  313  314  315  316  317  318  319  320  321  322  323  324  325  326  327  328  329  330  331  332  333  334  335  336  337  338  339  340  341  342  343  344  345  346  347  348  349  350  351  352  353  354  355  356  357  358  359  360  361  362  363  364  365  366  367  368  369  370  371  372  373  374  375  376  377  378  379  380  381  382  383  384  385  386  387  388  389  390  391  392  393  394  395  396  397  398  399  400  401  402  403  404  405  406  407  408  409  410  411  412  413  414  415  416  417  418  419  420  421  422  423  424  425  426  427  428  429  430  431  432  433  434  435  436  437  438  439  440  441  442  443  444  445  446  447  448  449  450  451  452  453  454  455  456  457  458  459  460  461  462  463  464  465  466  467  468  469  470  471  472  473  474  475  476  477  478  479  480  481  482  483  484  485  486  487  488  489  490  491  492  493  494  495  496  497  498  499  500  501  502  503  504  505  506  507  508  509  510  511  512  513  514  515  516  517  518  519  520  521  522  523  524  525  526  527  528  529  530  531  532  533  534  535  536  537  538  539  540  541  542  543  544  545  546  547  548  549  550  551  552  553  554  555  556  557  558  559  560  561  562  563  564  565  566  567  568  569  570  571  572  573  574  575  576  577  578  579  580  581  582  583  584  585  586  587  588  589  590  591  592  593  594  595  596  597  598  599  600  601  602  603  604  605  606  607  608  609  610  611  612  613  614  615  616  617  618  619  620  621  622  623  624  625  626  627  628  629  630  631  632  633  634  635  636  637  638  639  640  641  642  643  644  645  646  647  648  649  650  651  652  653  654  655  656  657  658  659  660  661  662  663  664  665  666  667  668  669  670  671  672  673  674  675  676  677  678  679  680  681  682  683  684  685  686  687  688  689  690  691  692  693  694  695  696  697  698  699  700  701  702  703  704  705  706  707  708  709  710  711  712  713  714  715  716  717  718  719  720  721  722  723  724  725  726  727  728  729  730  731  732  733  734  735  736  737  738  739  740  741  742  743  744  745  746  747  748  749  750  751  752  753  754  755  756  757  758  759  760  761  762  763  764  765  766  767  768  769  770  771  772  773  774  775  776  777  778  779  780  781  782  783  784  785  786  787  788  789  790  791  792  793  794  795  796  797  798  799  800  801  802  803  804  805  806  807  808  809  810  811  812  813  814  815  816  817  818  819  820  821  822  823  824  825  826  827  828  829  830  831  832  833  834  835  836  837  838  839  840  841  842  843  844  845  846  847  848  849  850  851  852  853  854  855  856  857  858  859  860  861  862  863  864  865  866  867  868  869  870  871  872  873  874  875  876  877  878  879  880  881  882  883  884  885  886  887  888  889  890  891  892  893  894  895  896  897  898  899  900  901  902  903  904  905  906  907  908  909  910  911  912  913  914  915  916  917  918  919  920  921  922  923  924  925  926  927  928  929  930  931  932  933  934  935  936  937  938  939  940  941  942  943  944  945  946  947  948  949  950  951  952  953  954  955  956  957  958  959  960  961  962  963  964  965  966  967  968  969  970  971  972  973  974  975  976  977  978  979  980  981  982  983  984  985  986  987  988  989  990  991  992  993  994  995  996  997  998  999  1000  1001  1002  1003  1004  1005  1006  1007  1008  1009  1010  1011  1012  1013  1014  1015  1016  1017  1018  1019  1020  1021  1022  1023  1024  1025  1026  1027  1028  1029  1030  1031  1032  1033  1034  1035  1036  1037  1038  1039  1040  1041  1042  1043  1044  1045  1046  1047  1048  1049  1050  1051  1052  1053  1054  1055  1056  1057  1058  1059  1060  1061  1062  1063  1064  1065  1066  1067  1068  1069  1070  1071  1072  1073  1074  1075  1076  1077  1078  1079  1080  1081  1082  1083  1084  1085  1086  1087  1088  1089  1090  1091  1092  1093  1094  1095  1096  1097  1098  1099  1100  1101  1102  1103  1104  1105  1106  1107  1108  1109  1110  1111  1112  1113  1114  1115  1116  1117  1118  1119  1120  1121  1122  1123  1124  1125  1126  1127  1128  1129  1130  1131  1132  1133  1134  1135  1136  1137  1138  1139  1140  1141  1142  1143  1144  1145  1146  1147  1148  1149  1150  1151  1152  1153  1154  1155  1156  1157  1158  1159  1160  1161  1162  1163  1164  1165  1166  1167  1168  1169  1170  1171  1172  1173  1174  1175  1176  1177  1178  1179  1180  1181  1182  1183  1184  1185  1186  1187  1188  1189  1190  1191  1192  1193  1194  1195  1196  1197  1198  1199  1200  1201  1202  1203  1204  1205  1206  1207  1208  1209  1210  1211  1212  1213  1214  1215  1216  1217  1218  1219  1220  1221  1222  1223  1224  1225  1226  1227  1228  1229  1230  1231  1232  1233  1234  1235  1236  1237  1238  1239  1240  1241  1242  1243  1244  1245  1246  1247  1248  1249  1250  1251  1252  1253  1254  1255  1256  1257  1258  1259  1260  1261  1262  1263  1264  1265  1266  1267  1268  1269  1270  1271  1272  1273  1274  1275  1276  1277  1278  1279  1280  1281  1282  1283  1284  1285  1286  1287  1288  1289  1290  1291  1292  1293  1294  1295  1296  1297  1298  1299  1300  1301  1302  1303  1304  1305  1306  1307  1308  1309  1310  1311  1312  1313  1314  1315  1316  1317  1318  1319  1320  1321  1322  1323  1324  1325  1326  1327  1328  1329  1330  1331  1332  1333  1334  1335  1336  1337  1338  1339  1340  1341  1342  1343  1344  1345  1346  1347  1348  1349  1350  1351  1352  1353  1354  1355  1356  1357  1358  1359  1360  1361  1362  1363  1364  1365  1366  1367  1368  1369  1370  1371  1372  1373  1374  1375  1376  1377  1378  1379  1380  1381  1382  1383  1384  1385  1386  1387  1388  1389  1390  1391  1392  1393  1394  1395  1396  1397  1398  1399  1400  1401  1402  1403  1404  1405  1406  1407  1408  1409  1410  1411  1412  1413  1414  1415  1416  1417  1418  1419  1420  1421  1422  1423  1424  1425  1426  1427  1428  1429  1430  1431  1432  1433  1434  1435  1436  1437  1438  1439  1440  1441  1442  1443  1444  1445  1446  1447  1448  1449  1450  1451  1452  1453  1454  1455  1456  1457  1458  1459  1460  1461  1462  1463  1464  1465  1466  1467  1468  1469  1470  1471  1472  1473  1474  1475  1476  1477  1478  1479  1480  1481  1482  1483  1484  1485  1486  1487  1488  1489  1490  1491  1492  1493  1494  1495  1496  1497  1498  1499  1500  1501  1502  1503  1504  1505  1506  1507  1508  1509  1510  1511  1512  1513  1514  1515  1516  1517  1518  1519  1520  1521  1522  1523  1524  1525  1526  1527  1528  1529  1530  1531  1532  1533  1534  1535  1536  1537  1538  1539  1540  1541  1542  1543  1544  1545  1546  1547  1548  1549  1550  1551  1552  1553  1554  1555  1556  1557  1558  1559  1560  1561  1562  1563  1564  1565  1566  1567  1568  1569  1570  1571  1572  1573  1574  1575  1576  1577  1578  1579  1580  1581  1582  1583  1584  1585  1586  1587  1588  1589  1590  1591  1592  1593  1594  1595  1596  1597  1598  1599  1600  1601  1602  1603  1604  1605  1606  1607  1608  1609  1610  1611  1612  1613  1614  1615  1616  1617  1618  1619  1620  1621  1622  1623  1624  1625  1626  1627  1628  1629  1630  1631  1632  1633  1634  1635  1636  1637  1638  1639  1640  1641  1642  1643  1644  1645  1646  1647  1648  1649  1650  1651  1652  1653  1654  1655  1656  1657  1658  1659  1660  1661  1662  1663  1664  1665  1666  1667  1668  1669  1670  1671  1672  1673  1674  1675  1676  1677  1678  1679  1680  1681  1682  1683  1684  1685  1686  1687  1688  1689  1690  1691  1692  1693  1694  1695  1696  1697  1698  1699  1700  1701  1702  1703  1704  1705  1706  1707  1708  1709  1710  1711  1712  1713  1714  1715  1716  1717  1718  1719  1720  1721  1722  1723  1724  1725  1726  17</p>		

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

PROCESS FOR THE PRODUCTION OF A PROTEINField of the Invention

This invention relates to a process for the production of a protein, particularly a metalloproteinase by a recombinant DNA process. The invention also relates to specific proteins, DNA sequences, vectors, host organisms and to pharmaceutical compositions.

Background to the Invention

The metalloproteinases are a family of enzymes produced in mammalian tissues and which are believed to play an important role in the resorption of the extracellular matrices of connective tissues.

The family of metalloproteinases includes collagenase, gelatinase and stromelysin which work together synergistically to digest all the major macromolecules comprising extracellular matrices. The enzymes are found in latent proenzyme form activatable by trypsin or 4-aminophenylmercuric acetate (APMA). The metalloproteinases have a zinc atom at their active site and require calcium for full activity.

Usually tissue metalloproteinases are coordinately synthesised, and for many tissues a specific stimulus is needed to induce synthesis (Reynolds J. Brit. J. Dermatology (1985) 112 715-723). The metalloproteinases are present naturally in low concentration and cannot be extracted in large amounts from mammalian tissues.

Of particular interest is stromelysin which is known to degrade many components of the connective tissues such as proteoglycan core protein, non-helical regions of type IV collagen, laminin, fibronectin, elastin, gelatin and procollagens types I, II and III and which may play a role in biological processes such as wound

healing. Stromelysin may be useful therapeutically in processes such as debridement of dermal ulcers and modifying scar tissue formation arising from the healing of wounds such as burns or necrosis. Stromelysin may also be used in the treatment of herniated vertebral discs to effect dissolution of nucleus pulposus. In order to use stromelysin in this way it must be produced on a large scale and at a commercially viable cost. We provide mammalian stromelysin in such commercially worthwhile amounts by producing it in accordance with the invention, using recombinant DNA techniques.

Preprostromelysin is a large molecular weight form of stromelysin which on secretion is processed to yield the lower molecular weight prostromelysin. Activation of prostromelysin by, for example, treatment with 4-amino phenylmercuric acetate yields the mature biologically active stromelysin.

#### Summary of the Invention

According to the present invention there is provided a process for the production of a mammalian stromelysin comprising culturing host cells transformed with a DNA sequence coding for the stromelysin.

The term mammalian stromelysin as used herein denotes a mammalian stromelysin having the amino acid sequence of an authentic mammalian stromelysin, an analogue thereof, or a biologically active peptide fragment of either of these, having the biological activity associated with authentic mammalian stromelysin.

In a preferred embodiment the invention provides a process for the production of human stromelysin comprising culturing host cells transformed with a DNA sequence coding for human stromelysin.

The process allows the production of relatively large quantities of stromelysin and, for the first time, facilitates complete characterisation of the structure and pharmacological properties of the protein.

The stromelysin preferably has an amino acid sequence of greater than 90 percent homology (common amino acids/total amino acids) with the sequence of amino acids 1 to 477 of human preprostromelysin as shown in Figure 4 of the accompanying drawings. More preferably, the homology is greater than 98 per cent and most preferably the human preprostromelysin has the amino acid sequence substantially as shown in Figure 4.

In addition to the complete mammalian stromelysin proteins, it is envisaged that relatively small peptide fragments of such proteins e.g. a peptide fragment of stromelysin may be useful as a metalloproteinase. Thus, in a particular embodiment of the first aspect of the invention, and where appropriate in subsequent aspects of the invention, the term stromelysin includes a peptide fragment of a stromelysin protein.

The mammalian stromelysin is suitably a methionine-stromelysin or a methionine-prostromelysin. It is currently understood that to obtain expression of a DNA sequence, the DNA sequence must possess a 5' ATG codon and the corresponding polypeptide therefore possesses an N-terminal methionine amino acid. As used herein the term "methionine-stromelysin" and "methionine-prostromelysin" denote an authentic mammalian stromelysin or prostromelysin, (or an authentic mammalian stromelysin or prostromelysin, modified or substituted to provide a functionally equivalent protein) having an N-terminal methionine residue. Preferably the methionine residue is adjacent to the N-terminal amino acid of the stromelysin or prostromelysin but may be separated therefrom by one or more amino acids provided that the protein possesses stromelysin or prostromelysin functional activity.

In a second aspect of the invention there is provided a process for the production of a mammalian stromelysin comprising producing a precursor of the stromelysin by culturing host cells transformed with a DNA sequence coding for the stromelysin precursor and cleaving the precursor to produce the stromelysin.

In a preferred embodiment of the second aspect of the invention

- 4 -

we provide a process for the production of human stromelysin comprising producing a precursor of human stromelysin by culturing host cells transformed with a DNA sequence coding for the stromelysin precursor and cleaving the precursor to produce human stromelysin.

5           The precursor protein may be a methionine-stromelysin or methionine-prostromelysin.

          The precursor protein may be a mammalian stromelysin or a prostromelysin with an amino terminal signal sequence. The signal sequence may be a sequence having the effect of encouraging  
10       transport of the expression products from the host cell in which the DNA sequences have been expressed. For example, in the case of human stromelysin, a signal sequence of amino acids such as depicted by amino acids 1 to 17 in Figure 4 of the accompanying drawings may be attached to the amino terminal amino acid to form  
15       preprostromelysin. This signal sequence assists in the export of the product from eukaryotic host cells and is itself cleaved from the product during transport through the cell membrane.

          The precursor protein may be a fusion protein comprising a heterologous protein and a mammalian stromelysin or prostromelysin  
20       protein. The heterologous protein may be all or a part of a protein capable of being produced in a host organism, preferably at a high level. Such heterologous proteins include  $\beta$ -galactosidase, chloramphenicol acetyl transferase (CAT) and the product of the trpE gene. The fusion protein preferably includes a site susceptible to  
25       selective chemical or enzymatic cleavage between the stromelysin or prostromelysin protein and the heterologous protein. The heterologous protein may be a yeast signal sequence and the host organism may be a yeast. In this preferred embodiment, the yeast host organism advantageously cleaves the fusion protein to produce a  
30       mature stromelysin or prostromelysin.

          The process of the invention permits the production of essentially pure human stromelysin, prostromelysin or preprostromelysin. The term essentially pure is used to denote stromelysin which is essentially free from other proteins of human origin.

- 5 -

In a third aspect the invention therefore provides an essentially pure mammalian stromelysin, or stromelysin or preprostromelysin characterised in that the mammalian stromelysin, prostromelysin or preprostromelysin is human stromelysin, prostromelysin or preprostromelysin.

In a preferred embodiment of the third aspect of the invention the essentially pure human stromelysin has substantially the amino acid sequence of from 100 to 477 as shown in Figure 4 of the accompanying drawings, the essentially pure human prostromelysin has the amino acid sequence of from 18 to 477 as shown in Figure 4 of the accompanying drawings and the essentially pure human preprostromelysin has the amino acid sequence of from 1 to 477 as shown in Figure 4 of the accompanying drawings.

In a fourth aspect of the invention, we provide a mammalian stromelysin or prostromelysin produced by the process of the first aspect of the invention or a mammalian stromelysin or prostromelysin precursor protein produced as an intermediate compound.

In a preferred embodiment of the fourth aspect of the invention the stromelysin is human stromelysin or prostromelysin or a precursor thereof.

In a fifth aspect of the invention, we provide a fusion protein comprising a heterologous protein and a mammalian stromelysin or prostromelysin.

In a preferred embodiment of the fifth aspect of the invention the fusion protein comprises a heterologous protein and human stromelysin or human prostromelysin.

In a sixth aspect of the invention, we provide a DNA sequence coding for the amino acid sequence of a mammalian stromelysin or prostromelysin, or a precursor thereof provided that when the DNA sequence codes for rat stromelysin the DNA sequence is not the pTR1 cDNA sequence described by Matrisian et al (EMBO Journal 4 14350-1440 (1985)). Preferably, we provide a DNA sequence coding

for human preprostromelysin having substantially the nucleotide sequence from 51 to 1484 inclusive as shown in Figure 4 of the accompanying drawings. Preferably we provide a DNA sequence coding for human prostromelysin having substantially the nucleotide sequence from 102 to 1484 inclusive as shown in Figure 4 of the accompanying drawings. Preferably we provide a DNA sequence coding for human stromelysin having substantially the nucleotide sequence from 348 to 1484 inclusive as shown in Figure 4 of the accompanying drawings.

10 In a seventh aspect of the invention, we provide an expression vector including a DNA sequence coding for a mammalian stromelysin, prostromelysin or precursor thereof. The vector is adapted for use in a given host cell by the provision of suitable selectable markers, promoters and other control regions as appropriate.

15 In a preferred embodiment of the seventh aspect of the invention the vector includes a DNA sequence coding for human stromelysin or prostromelysin or a precursor thereof.

In an eighth aspect of the invention, we provide host cells transformed with a vector according to the seventh aspect of the invention. The host cells may be any host organism which may be transformed with a vector including a DNA sequence coding for a mammalian stromelysin or a precursor thereof, such that expression of the DNA sequence occurs. Suitable host cells include yeasts (for example Saccharomyces cerevisiae) and mammalian cells in tissue culture (for example, hamster ovary or mouse mammary tumour cells). Preferably, where the host cell is a bacterium or a yeast, the vector includes a DNA sequence coding for methionine-stromelysin or methionine prostromelysin or a fusion protein including a stromelysin or prostromelysin and when the host cell is a mammalian cell in tissue culture, the vector preferably includes a DNA sequence coding for a prestromelysin or a preprostromelysin.

In a ninth aspect of the invention, we provide a pharmaceutical composition comprising a mammalian stromelysin preferably human stromelysin and a pharmaceutically acceptable



- 7 -

excipient. The pharmaceutical composition may be, for example, in the form of an injectable solution or in a form suitable for topical administration. The pharmaceutical composition may contain a stromelysin or a prostromelysin preferably human stromelysin or  
5 human prostromelysin which may be activated in vitro or in vivo to give biologically active stromelysin.

In a preferred embodiment of the ninth aspect of the invention we provide a pharmaceutical composition containing an effective amount of a mammalian stromelysin or prostromelysin, preferably  
10 human stromelysin or prostromelysin, for use in the debridement of dermal ulcers, modification of scar tissue formation arising from the healing of wounds such as burns and necrosis and in the treatment of herniated vertebral discs.

In a tenth aspect of the invention we provide a process for the  
15 production of a pharmaceutical composition comprising bringing a mammalian stromelysin or prostromelysin, preferably human stromelysin or prostromelysin into association with a pharmaceutically acceptable carrier.

In an eleventh aspect the invention provides a method of  
20 therapy comprising treating a patient with an effective amount of a mammalian stromelysin preferably human stromelysin, for instance for debridement of dermal ulcers, modification of scar tissue formation arising from the healing of wounds such as burns and necrosis or treatment of herniated vertebral discs.

25 Preferably, the stromelysin produced in accordance with the invention will have a useful pharmacological effect without significant antigenic reaction with the immune system. In particular the compounds may be used in the debridement of dermal ulcers, modification of scar tissue formation arising from the  
30 healing of wounds such as burns and necrosis and in the treatment of herniated vertebral discs.

The DNA sequence sequence coding for human stromelysin as described in Figure 4 may be used to design DNA probes for use in

identifying the over expression of stromelysin indicative of connective tissue disease states and the invention extends to such DNA probes.

5 In a twelvth aspect the invention provides a DNA hybridisation probe comprising a sequence of nucleotides selected from the nucleotide sequence from 1 to 1810 as shown in Figure 4 of the accompanying drawings.

10 In a further aspect the invention provides an antibody having specificity for an antigenic determinant of a mammalian stromelysin. The antibody may be a polyclonal or monoclonal antibody. The antibody may be labelled with a detectable label.

Stromelysin is shown to be able to enhance the activity of collagenase after its activation with, for example, trypsin or APMA and the invention extends to the use of stromelysin for this purpose.

15 Brief Description of the Drawings

The invention is further described by the following non-limiting examples which refer to the accompanying diagrams Figures 1 to 9 and Tables 1 and 2, short particulars of which are given below.

20 Figure 1 shows the N. terminal amino acid sequence of rabbit APMA activated stromelysin (a) and the oligo-nucleotide probe based on this sequence. The 50 base oligonucleotide probe was made in two 25 base halves (b and c) and the complementary 26-mer (d) was synthesised in  
25 order to facilitate their ligation. The numbering of the amino acids refers to their position in the cDNA predicted rabbit stromelysin sequence (see Fig 2)

30 Figure 2 Shows the partial nucleotide sequence of rabbit stromelysin and the predicted amino acid sequence

- Figure 3 Shows the restriction maps of 3 human stromelysin cDNAs
- Figure 4 Shows the restriction map and nucleotide sequence of human stromelysin cDNA and the predicted amino acid sequence
- 5
- Figure 5 Shows electrophoretic analysis of the products of activation of stromelysin purified from:
- panel A - human gingival fibroblast culture medium.
- 10 panel B - that secreted by C127 cells transfected with stromelysin-containing vector
- panel C - that secreted by COS cells transfected with stromelysin-containing vector
- Figure 6 Shows a graph of the time course of activation of C127 cell secreted prostromelysin by various concentrations of trypsin or APMA
- 15
- Figure 7 Shows electrophoretic analysis of the products of C127 prostromelysin activation with APMA
- Figure 8 Shows a graph of the effect of different amounts of purified human fibroblast prostromelysin or prostromelysin secreted by C127 cells on the activation by trypsin of:
- 20
- A). procollagenase secreted by transfected C127 cells, and
- 25 B). procollagenase purified from human gingival fibroblast culture
- Figure 9 Shows electrophoretic analysis of procollagenase from human gingival fibroblast culture medium activated by APMA, APMA + stromelysin, trypsin, or trypsin + stromelysin.
- 30

Table 1      Amino acid composition of human prostromelysin

Table 2      Amino acid composition of human stromelysin

Detailed Description of the Embodiments

5            In the following example, prostromelysin is activated by treatment with 4-amino phenylmercuric acetate to yield mature stromelysin. It will be appreciated, however, that by using alternative methods of cleavage of the proenzyme such as enzymic digestion, different forms of active stromelysin may be produced and these are included within the scope of the present invention.

10          1.1 THE ISOLATION OF A RABBIT cDNA ENCODING STROMELYSIN

            4-aminophenylmercuric acetate (APMA) activated (Cawston and  
Murphy in: Methods in Enzymology, Volume 80 pp711-722 (1981))  
rabbit fibroblast stromelysin was purified from the culture media of  
calvariae (Galloway, W. A. et al Biochem. J. 209, 741-752 (1983)).  
15          It was reduced and carboxymethylated and subjected to N-terminal  
sequencing by automated Edman degradation on an Applied Biosystems  
gas-phase sequenator. The 50 base oligonucleotide probe was  
designed according to previously described rules to be capable  
theoretically of hybridizing to both rabbit stromelysin and  
20          collagenase cDNAs (Grantham R. et al Nucleic Acids Res. 9, r43-r47  
(1981) and Lathe, R. J. Molec. Biol. 183, 12252-12258 (1983)). It  
was synthesized as two continuous 25 mers (b and c, Figure 1) by  
automated solid-phase phosphotriester chemistry followed by  
purification by HPLC (Patel T. P. et al Nucleic Acids Res. 10,  
25          5605-5619 (1982)). A complementary 26 mer (d) was also made (see  
Fig. 1).

            Rabbit fibroblasts from explants of normal synovium were grown  
to confluence in Dulbecco's modified Eagle's medium (DMEM)  
supplemented with 10% fetal calf serum (FCS). After washing they  
30          were then maintained in serum-free media for 48 h in the presence of

50-200 ng ml<sup>-1</sup> 12 - 0 - tetradecanoylphorbol - 13 - acetate (TPA). mRNA was isolated from approximately 5.10<sup>7</sup> cells by the guanidinium isothiocyanate/hot phenol method (Maniatis et al 1982 in. Molecular Cloning: A laboratory Manual; Cold Spring Harbor laboratory, New York), followed by oligo (dT)-cellulose column chromatography (Aviv and Leder, 1972 PNAS USA 69 1408-1412). Standard procedures were used to synthesise cDNA (Gubler and Hoffman, 1983 Gene 25 263-269) and a library of 75,000 plaques was established by using EcoRI linkers to ligate the cDNA into  $\lambda$  gt10 essentially as described by Huynh et al in DNA Cloning Vol 1 ed. D. M. Glover, IRL Press p49-78.

The oligonucleotide probe was prepared by first subjecting to a kinase reaction the 25 mer labelled (c) fig 1 and then ligating it to the 25-mer labelled (b) in the presence of the complementary 26-mer (d). The 50-base oligonucleotide probe resulting from the ligation was purified from a denaturing polyacrylamide gel (Maxam A. M & Gilbert W. in Methods in Enzymology, Volume 65, pp 499-560 (1980)) and then kinased to a specific activity of approximately 3.10<sup>8</sup> Ce cpm  $\mu$ g<sup>-1</sup>.

The plaques were transferred to nitrocellulose filters, denatured, and then prehybridised in 5 x SSC, 5 x Denhardt's solution, 50mM NaH<sub>2</sub>PO<sub>4</sub>, 100  $\mu$ gml denatured salmon sperm DNA for 6h at 60°C. Hybridisation was then undertaken for 16h at 40°C in an identical buffer to which had been added 0.1% SDS and the probe DNA at approximately 4ng ml<sup>-1</sup>. The filters were then washed in 6 X SSC before a final stringent wash (1 X SSC, 0.1% SDS for 15 min at 50°C) followed by autoradiography for 16 h with an intensifying screen. 10 putatively positive plaques were identified of which one gave positive signals after a second screening undertaken as described above. DNA was isolated and the cDNA insert was subcloned into plasmid pSP64 (Melton D. A. et al Nucleic Acids Res. 12, 7035-7056 (1984)). The complete nucleotide sequence of the cDNA was determined by the dideoxy method (Sanger F., Nicklen S. & Coulson A. R. Proc. Natl. Acad. Sci USA 74 5463-5467 (1977)) after subcloning restriction fragments into M13 and transformation into JM101 (Messing J. & Vieira, J. Gene 19 269-276 (1982)).

- 12 -

As shown in fig 2 the cDNA is 532 nucleotides in length and although lacking a poly A tract does have an open reading frame encoding 170 amino acids. Residues 101-127 are identical with the N-terminal amino acid sequence of rabbit stromelysin (Fig 1a) and the amino acid sequence encoded by the cDNA therefore represents the N-terminal third of rabbit stromelysin. This cDNA was therefore used as a probe to identify the human stromelysin cDNA. It was labelled with  $^{32}\text{P}$   $\alpha$  dATP and Klenow fragment using the random hexanucleotide method (Feinberg and Vogelstein, 1983, Anal. Biochem, 132 6-13) to a specific activity of  $2.10^7$  Ce cpm  $\mu\text{g}^{-1}$ .

## 1.2 ISOLATION OF A HUMAN cDNA ENCODING STROMELYSIN

Human fibroblasts derived from gingival explants (passage 3 to 6) were cultivated in DMEM supplemented with 0.2% lactalbumin hydrolysate and 5% partially purified pig IL-1. After 48h mRNA was isolated from approximately  $5 \times 10^8$  cells by the guanidinium isothiocyanate/CsCl method (Maniatis *et al* 1982 in Molecular Cloning: A laboratory Manual; Cold Spring Harbor Laboratory, New York). The mRNA was used to generate a library of 200,000 recombinant plaques in  $\lambda\text{gt}10$  essentially as described above. The plaques were transferred to nitrocellulose, denatured, and then prehybridised in 6 X SSC, 50% formamide, 5 X Denhardt's solution,  $100 \mu\text{g ml}^{-1}$  denatured salmon sperm DNA for 16 h at  $42^\circ\text{C}$ . They were then hybridised for 48h at  $42^\circ\text{C}$  in an identical buffer to which had been added 0.1% SDS and the rabbit stromelysin probe cDNA at a final concentration of approximately  $17 \text{ ng ml}^{-1}$ . The filters were then washed with the highest stringency being 2 X SSC, 0.1% SDS at  $42^\circ\text{C}$  for 30 minutes before autoradiography with an intensifying screen for 48h. 21 putatively positive plaques were identified of which 16 gave a clear positive signal after a second screening undertaken as described above. The cDNA insert of representative plaques was isolated and subcloned into plasmid pSP64 (Melton D. A. *et al* Nucleic Acids Res. 12, 7035-7056 (1984)). Restriction mapping of the largest inserts confirmed that they were related overlapping DNA segments (Fig. 3) and their nucleotide sequence was compiled by the dideoxy method (Sanger F., Nicklen S. &

Coulson A. R. Proc. Natl. Acad. Sci USA 74 5463-5467 (1977)) after subcloning restriction fragments into M13 and transformation into JM101 (Messing J. & Vieira, J. Gen. 19, 269-276 (1982)). This information is shown in Fig 4, where the solid arrows indicate direction of dideoxy sequencing and the broken arrows show directions of sequencing by the Maxam and Gilbert method (Maxam, A.M. and Gilbert, W., Methods Enzymol. (1980) 65 499-560).

### 1.3 IDENTIFICATION OF RAT cDNA ENCODING STROMELYSIN

We have discovered a high degree of homology between rabbit stromelysin and another amino acid sequence predicted from a rat cDNA, referred to as pTR1 (Matrisian L. M. et al EMBO Journal 4, 1435-1440 (1985)). This cDNA corresponds to a mRNA which is highly enriched in normal fibroblasts after infection with either polyoma virus or rous sarcoma virus or transfection with either the middle t oncogene or the cellular oncogene H-ras. The same mRNA was also reported to be specifically induced after exposure of fibroblasts to EGF (Matrisian L. M. et al EMBO Journal 4, 1435-1440 (1985)). The predicted Mr of the rat protein encoded by pTR1 is approximately 53000, in close agreement with the in vitro translation product of rabbit stromelysin mRNA (Frisch S. M., Chin J. R. & Werb Z. J. Cell Biol. 97, (2, Pt. 5): 430a (Abstr.) (1983)), and data not shown]. We conclude therefore, that the protein encoded by pTR1 is rat stromelysin. The identification of pTR1 as rat stromelysin allows, for the first time, the production of rat stromelysin using recombinant DNA techniques.

### 2. NUCLEOTIDE SEQUENCE OF HUMAN STROMELYSIN cDNA AND THE PREDICTED AMINO ACID SEQUENCE

The cDNA is 1825 nucleotides in length and appears to be complete at the 3' end because it has a poly A tail preceded by the polyadenylation signal AATAAA (Proudfoot and Brownlee, 1981, Nature, 252 359-362). It contains an open reading frame (nucleotides 51 to 1481) which translates into a polypeptide of 477 amino acids. Evidence that the cDNA encodes stromelysin comes from the finding that 24 of the 27 amino acids encoded by nucleotides 348 to 428 are

identical with and in the same position as the 27 amino acids identified by N-terminal sequencing the APMA activated rabbit stromelysin (Fig 1a and Fig 4). Furthermore most of the amino acids in the N-terminal third of the molecule are identical with and in the same position as those amino acids predicted from the rabbit partial stromelysin cDNA.

Hydrophobicity plots suggest that human stromelysin is rather water soluble except for a hydrophobic N-terminal sequence of 17 amino acids. This is consistent with these amino acids being the hydrophobic core of a signal sequence which is cleaved during secretion to liberate mature protein. Since it is thought that the metalloproteinases are secreted as proenzymes (Harris E. D. *et al* Collagen Rel Res 4 493-512 (1984)) we conclude that the amino acids found between the signal sequence and the phenylalanine (F) at the N-termini of the APMA - activated form of rabbit stromelysin (residue 100 in the human stromelysin sequence) are cleaved off during activation. Preprostromelysin is therefore considered to be comprised of amino acids 1-477, prostromelysin is considered to be comprised of amino acids 18-477 and stromelysin is considered to be comprised of amino acids 100-477. The amino acid composition of prostromelysin and stromelysin together with the molecular weight of the core proteins is shown in tables 1 and 2 respectively. Potential glycosylation sites within the sequences exist, and it is envisaged that expression of these proteins in appropriate cells (see below) may give rise to glycosylated products.

### 3. THE PRODUCTION OF STROMELYSIN

#### 3.1 EXPRESSION IN E. COLI

The expression of stromelysin or prostromelysin with an additional NH<sub>2</sub>-terminal methionine residue (met-stromelysin or met-prostromelysin) in *E. coli* can be achieved by using oligonucleotide linkers to join the mature stromelysin or prostromelysin encoding sequence described in Section 2 above and shown in Figure 4 with a promoter, Shine Dalgarno sequence and an initiating ATG codon. The *E. coli* *trpE* promoter and Shine Dalgarno sequence may be used.



TABLE 1Amino Acid Composition of Human Prostromelysin

	<u>Residue</u>	<u>Number</u>	<u>%</u>	<u>Weight</u>	<u>%</u>
	F	32	6.96	4704	9.02
	L	38	8.26	4294	8.23
5	I	20	4.35	2260	4.33
	M	6	1.30	786	1.51
	V	28	6.09	2772	5.31
	S	27	5.87	2349	4.50
	P	36	7.83	3492	6.69
10	T	27	5.87	2727	5.23
	A	27	5.87	1917	3.68
	Y	17	3.70	2771	5.31
	H	13	2.83	1781	3.41
	Q	9	1.96	1152	2.21
15	N	16	3.48	1824	3.50
	K	32	6.96	4096	7.85
	D	36	7.83	4140	7.94
	E	30	6.52	3870	7.42
	C	3	0.65	309	0.59
20	W	8	1.74	1488	2.85
	R	23	5.00	3588	6.88
	G	32	6.96	1824	3.50
	TOTAL	460		52162	

TABLE 2Amino Acid Composition of Human Stromelysin

	<u>Residue</u>	<u>Number</u>	<u>%</u>	<u>Weight</u>	<u>%</u>
5	F	30	7.94	4410	10.31
	L	30	7.94	3390	7.92
	I	19	5.03	2147	5.02
	M	3	0.79	393	0.92
	V	19	5.03	1881	4.40
10	S	24	6.35	2088	4.88
	P	32	8.47	3104	7.25
	T	24	6.35	2424	5.67
	A	25	6.61	1775	4.15
	Y	13	3.44	2119	4.95
15	H	12	3.17	1644	3.84
	Q	6	1.59	768	1.79
	N	14	3.70	1596	3.73
	K	22	5.82	2816	6.58
	D	28	7.41	3220	7.53
20	E	25	6.61	3225	7.54
	C	2	0.53	206	0.48
	W	8	2.12	1488	3.48
	R	17	4.50	2652	6.20
	G	25	6.61	1425	3.33
TOTAL		378		42789	

These sequences are present for example on plasmid pCT54 into which DNA sequences encoding proteins of interest can be inserted in a Clal site (Emtage et al (1983), Proc. Nat. Acad. Sci. USA, 80, 3671-3675). However, other plasmids also harbouring these or other promoter and Shine Dalgarno sequences but which are amplifiable and from which expression can be tightly regulated may also be used (see, for example, European patent application EP-A2-0121386). The stromelysin or prostromelysin coding sequence without the signal coding sequence but with an additional N-terminal methionine codon, may be inserted into the E. coli expression vector pMG196. This may be achieved by standard recombinant DNA techniques (Maniatis et al (1982), In: Molecular Cloning: A Laboratory Manual, 390-433). Expression of stromelysin or prostromelysin may also be achieved using analogous techniques to those described for expression of TIMP in European patent application No. 86300042.8.

### 3.2 PURIFICATION OF STROMELYSIN FROM E. COLI CELLS

E. coli cells harbouring a plasmid constructed as described above, when grown under optimal conditions for expression, will produce met-stromelysin or met-prostromelysin at levels of up to 10% of total cellular protein.

The soluble protein fraction from crude E. coli extracts is assayed for stromelysin activity essentially as described by Galaway et al (1983) Biochem. J. 209 741-752. A part or whole of the met-stromelysin expressed in E. coli may be in an insoluble form and therefore not detected in the above assay. In such circumstances, it is solubilised and activated prior to assay and purification. One example of how this can be achieved in relation to methionine-prochymosin production is described in our co-pending International patent application PCT/GB 83/00152 (published as WO 83/04418) and in published British patent application GB 2100737A. Having obtained soluble, active, partially purified stromelysin it is further purified using standard protein purification techniques, as described for example by Galaway et al (1983) Biochem. J. 209 741-752.

### 3.3 EXPRESSION IN YEAST

Using standard recombinant DNA techniques in a similar manner to those used to achieve expression in E. coli, plasmid vectors suitable for expression of stromelysin in yeast are constructed.

5 The constructions are based, for example, on the vectors described in co-pending published European patent application EP-A2-0073635. They contain the stromelysin or prostromelysin encoding sequence flanked by the yeast phosphoglycerate kinase (PGK) promoter and the PGK gene 3' untranslated end. The orientation of the stromelysin or

10 prostromelysin cDNA with respect to the PGK promoter is such that it ensures expression of mature stromelysin or prostromelysin with an additional NH<sub>2</sub>-terminal methionine residue as described for expression in E. coli. Alternatively, pre-stromelysin or prostromelysin may be expressed by joining the stromelysin or

15 prostromelysin encoding sequence to the vector such that the stromelysin signal sequence is left in place. Expression of a fusion protein between a yeast signal sequence (for example, the yeast  $\alpha$ -Factor signal sequence (Kurjan and Herskowitz, (1982), Cell, 30, 933-943), and mature stromelysin or prostromelysin can be

20 achieved through the use of appropriate linkers. These plasmid DNAs are introduced into yeast cells, for example, by the method of Beggs (Nature, (1978), 275, 104-109).

### 3.4 PURIFICATION OF STROMELYSIN FROM YEAST CELLS

Yeast cells containing these plasmids, when grown under optimal

25 conditions for stromelysin expression, will produce up to 5% of total cellular protein as stromelysin or prostromelysin. Depending on the alternatives described above, stromelysin or prostromelysin may or may not be secreted from the yeast cells. Expressed stromelysin is quantified, assayed and purified essentially as

30 described above if produced intercellularly. If secreted it is purified from cell supernatants by standard protein purification techniques (Galaway et al (1983) Biochem. J. 209 741-752).

### 3.5 EXPRESSION OF STROMELYSIN IN CULTURED ANIMAL CELLS

The preprostromelysin encoding cDNA was inserted into the different kinds of expression vector designed specifically for: 1. transient expression in COS cells (Gluzman, Y. (1981) Cell 23 175-182) and 2. Stable expression in C127 cells (Lowy, D.R. et al J. Virol. (1978) 26 291-298). The necessary DNA manipulations employed standard techniques (Maniatis et al In. Molecular Cloning, A Laboratory Manual, 1982, Cold Spring Harbor Laboratory, New York). The cDNA was tailored for insertion into the vectors by first converting the Ava I site encoded by nucleotides 1591-1596 (Figure 4) into an EcoR1 site. This was achieved by cleaving the cDNA with Ava I, filling in with T4 polymerase followed by addition of an EcoR1 linker. On cleavage with EcoR1 a 1600 bp EcoR1 fragment encoding preprostromelysin extending from the 5' EcoR1 site encoded by nucleotides 1-6 in Figure 4 to the newly created EcoR1 site is generated. This fragment was purified from a 1% low gelling temperature agarose gel and then ligated between the SV40 late promoter and the SV40 early polyadenylation regulatory elements in a "poison minus" derivative of a pBR322 COS cell vector (Lusky, M and Botchan, M.R., Nature, 1981, 293 79-81). Vector DNA containing the preprostromelysin sequence in the appropriate orientation with respect to the SV40 late promoter was purified from cesium chloride gradients and transfected into COS cells using DEAE dextran (Lopata, M.A. et al Nucleic Acids Res., 1984, 12 5707-5717). Serum-free supernatants were harvested 72 hours after transfection.

For expression in C127 cells the preprostromelysin sequence with the early SV40 polyadenylation regulatory elements was removed on a PstI to BamHI fragment from the COS cell vector and put under the control of the mouse metallothionein I promoter in a bovine papilloma virus (BPV) based vector. This arrangement was essentially as described for the expression of tissue inhibitor of metalloproteinase (TIMP), Docherty et al Nature 1985, 318 66-69 and UK Patent Application GB 2169295A. The BPV vector encoding preprostromelysin was introduced into C127 cells by the calcium phosphate coprecipitation method (Wigler, M. et al 1978, Cell 14 725-731) and CdCl<sub>2</sub> (20μM) - and ZnCl<sub>2</sub> (20μM) - resistant

foci were selected. Stromelysin producing cell lines were identified after 21 days and used to generate serum-free culture medium.

#### 4. STROMELYSIN ACTIVATION, ASSAY AND ELECTROPHORETIC ANALYSIS

5           The stromelysin produced as described above was activated with either trypsin or APMA. Trypsin was incubated with the stromelysin at ratios such as 1:100 to 1:1 respectively followed by the addition of a ten-fold excess of soya-bean trypsin inhibitor. Concentrations of trypsin such as 0.1 to 10  $\mu\text{g ml}^{-1}$  were employed and the  
10           temperatures and time of incubation were as described below. Treatment with 4-aminophenylmercuric acetate (APMA) was at 1 to 2 mM for various times as indicated below, at 37°C. Stromelysin activity was assayed using  $^{14}\text{C}$ -acetylated casein (Galloway, W.A., et al Biochem. J. 1983, 209 741-752) at 35°C for 1 or 4 h. 1 unit  
15           of stromelysin degrades 1  $\mu\text{g}$  of casein  $\text{min}^{-1}$  at 37°C.

          Stromelysin before and after activation as described above and in more detail below was treated with 20mM EDTA, reduced with 500mM 2-mercaptoethanol and run on 10% polyacrylamide mini gels in the presence of SDS. Gels were electro-transferred to nitrocellulose  
20           and the enzyme bands visualised using an antisera against rabbit stromelysin which was able to detect human stromelysin (Murphy, G. et al Collagen Rel. Res. 1986 6 351-364) and a peroxidase-labelled second antibody (Hembry, R.M., et al J. Cell Sci. 1985 73 105-119).

          As shown in Figure 5, prostromelysin preparations A, purified  
25           from human gingival fibroblast culture medium, B, secreted by C127 cells transfected with the stromelysin-containing vector and C, secreted by COS cells transfected with the stromelysin-containing vector, were treated as follows: 1. no treatment; 2. incubation with 1mM APMA for 2h at 37°C; 3. incubation with 10  $\mu\text{g/ml}$  trypsin  
30           for 30 min at 37°C; 4. as 3. with addition of soya bean trypsin inhibitor after 30 min and further incubation for 2h at 37°C; 5. incubation with 100  $\mu\text{g/ml}$  trypsin for 10 min at 4°C; 6. incubation with 10  $\mu\text{g/ml}$  trypsin for 5 min at 4°C;

7. as 6. with addition of soya bean trypsin inhibitor after 5 min and further incubation for 2h at 37°C. The samples were electrophoresed on a 10% polyacrylamide gel in SDS and reducing conditions, electro-blotted onto nitrocellulose and visualised using a sheep anti rabbit stromelysin antibody, and a peroxidase labelled rabbit anti sheep IgG.

As shown in Figure 6 prostromelysin secreted by C127 cells transfected with the stromelysin-containing vector was incubated with A, (●) 0.1µg/ml trypsin, (Δ) 1µg/ml trypsin, B, (□) 10µg/ml trypsin, C, (▽) 100µg/ml trypsin at 37°C (closed symbols) or 4°C (open symbols) in a volume of 20µl, or D, (◆) with 2mM APMA at 37°C. Activity elicited was assayed by degradation of <sup>14</sup>C-casein in a 1h assay at 37°C, after addition of soya bean trypsin inhibitor. Stromelysin incubated without trypsin or APMA had no activity.

Prostromelysin was activated with APMA (as described in Figure 6) for varying lengths of time. The samples were then treated with 20mM EDTA and electrophoresed under reducing conditions on a 10% polyacrylamide gel containing SDS and electroblotted and visualised as described in Fig. 5 and Methods. Figure 7 shows the SDS analysis where Track 1. 0 min; Track 2. 30 min; Track 3. 1h; Track 4. 2h; Track 5. 4h; Track 6. 15h.

The pro-forms of either purified natural stromelysin from human gingival fibroblasts, or the recombinant enzyme secreted by COS or C127 cells had an identical Mr of 57000, with a minor 60000 component (probably due to glycosylation (Nagase, H. et al (1983) *Biochem. J.* 214 281-288); Figure 5, lanes A1, B1, C1). These forms were completely inactive against casein or other substrates (Galloway, W.A. et al (1983) *Biochem. J.* 209 741-752) but could be activated by trypsin, (e.g. C127 stromelysin, Fig. 6A-C) or less efficiently by APMA, to degrade these substrates (Figure 6D). Trypsin activation was optimal over a wide range of concentrations; activation could be effected at 4°C, with marginally slower kinetics (Figure 6A-C). Only very high levels of trypsin inactivated stromelysin. Activation by APMA was slower, achieving 58% (COS) to 90% (C127 and

natural) of the trypsin value. Although maximum APMA activation was attained in 4-8 h at 37°C (Figure 6), it was found that activation was dependent upon stromelysin concentration (73% of optimal at a ten fold dilution and 33% at a one hundred fold dilution of a 50 unit/ml preparation). Hence, very low concentrations of enzyme, such as the COS cell culture media were probably not maximally activated even after 15 h incubation.

Analysis of the effect of these treatments on the prostromelysin by gel electrophoresis and immuno blotting showed that a reduction in Mr occurred to yield two major species of Mr. 50000 and 48000 slowly in the case of APMA (e.g. C127 stromelysin, Figure 7) and very rapidly in the case of trypsin (Figure 5 A3; B3,5; C3,5). At longer incubation times with APMA traces of an Mr. 28000 form of stromelysin were generated (Figure 7). Plasmin could also be used to generate bands of the same Mr as those produced by trypsin (data not shown). It was noted that optimal activation (as documented in Figure 6) did not require complete conversion of the upper doublet to the lower doublet (Figure 5; lanes A6, A7; Figure 3, lane 4). Using collagenase, Stricklin *et al* (Biochemistry 22 63-68 (1983)) have reported similar observations with activity detectable prior to an Mr loss. It has been proposed that conformational changes in the intact molecule occur, leading to subsequent self-cleavage. The activity of stromelysin elicited was 360 units/mg.

## 5. COLLAGENASE ACTIVATION ELECTROPHORETIC ANALYSIS AND ASSAY

The collagenase was activated as described for stromelysin or as detailed below. Electrophoretic analysis was undertaken as described for stromelysin except that enzyme bands were visualised using anti-human collagenase antisera followed by a peroxidase-labelled second antibody (Hembry, R.M. *et al* J. Cell Sci. 1985, 73 105-119). The collagenase was assayed by the <sup>14</sup>C-acetylated collagen diffuse fibril assay at 35°C for 4 hours (Whitham, S.E. *et al* Biochem. J. 1986, 240 913-916). 1 unit of collagenase degrades 1µg of type I collagen min<sup>-1</sup> at 35°C.



Figure 8 shows the results when procollagenase preparations, A, secreted by C127 cells transfected with the collagenase containing vector and B, purified from human gingival fibroblast culture medium were incubated with 10 $\mu$ g/ml trypsin at 37°C for 30 min in the presence of varying amounts of (●) purified human fibroblast prostromelysin or (■) prostromelysin secreted by C127 cells transfected with the stromelysin-containing vector, followed by the addition of excess soya bean trypsin inhibitor. Open symbols denote the effect of the corresponding activated stromelysin in the absence of trypsin. Activity was assayed using <sup>14</sup>C-type I collagen at 35°C for 4h. Results are expressed as the fold increase elicited by stromelysin relative to the activity with trypsin alone.

Figure 9 shows electrophoretic analysis of procollagenase purified from human gingival fibroblast culture medium treated as follows: 1. no treatment; 2. incubation with 1mM APMA at 37°C for 2h; 3. as 2, in the presence of purified stromelysin; 4. incubation with 10 $\mu$ g/ml<sup>-1</sup> trypsin at 37°C for 30 min, followed by addition of soya bean trypsin inhibitor; 5. as 4. in the presence of stromelysin.

Purified natural procollagenase has an Mr of 55000 with a minor component of Mr 59000 upon analysis by SDS polyacrylamide gel electrophoresis and blotting with a specific antiserum to collagenase (Figure 9; lane 1). The procollagenase could be activated by either APMA or trypsin treatment behaving precisely as described by Stricklin *et al* (Biochemistry 22 63-68 (1983)) with a fall in Mr of 10,000 (Figure 9, lanes 2 and 4). The activity elicited was very low, in the region of 320 units/mg. A similar observation was made by Vater *et al* (J. Biol. Chem. 258 9374-9382 (1983)) for rabbit procollagenase. However, the inclusion of either purified or recombinant human prostromelysin in the activation mixture enhanced the collagenase activity by up to 8 fold (Figure 8B). Similar effects of stromelysin on the molecular weight and activity of recombinant C127 cell derived human collagenase were also obtained. With the recombinant collagenase the stromelysin brought about a 12 fold enhancement in activity (Figure 8A).

5        Addition of previously activated stromelysin to the collagenase after trypsin treatment had less effect on the final collagenase activity detectable (data not shown). Active stromelysin alone elicited similar collagenase activities to those with trypsin alone. The ratio of stromelysin:collagenase for efficient activation was in the order of an excess of 2moles or more per mole. It is possible that higher specific activity stromelysin would be a more efficient activator.

10        Analysis of the changes occurring during these treatments showed that the procollagenase, underwent stepwise reductions in Mr APMA and trypsin generated two bands of about 50000 and 45000 (Figure 9, lanes 2 and 4) which were converted to bands of 48000 and 43000 in the presence of stromelysin (Figure 9; lanes 3 and 5). The same band patterns were generated by both natural and  
15        recombinant stromelysin. Activated stromelysin alone appeared to produce very small changes in procollagenase of Mr about 500, with limited further conversion to 48000 and 43000.

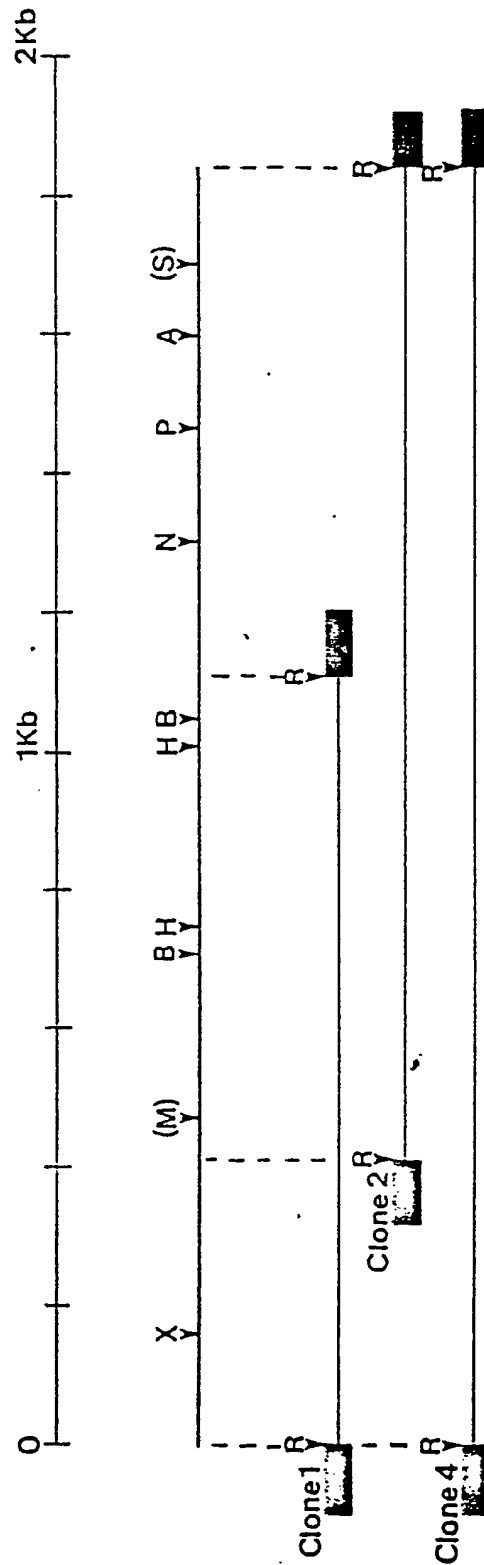
CLAIMS

1. A process for the production of a mammalian stromelysin comprising culturing host cells transformed with a DNA sequence coding for the stromelysin.
- 5 2. A process for the production of a mammalian stromelysin comprising producing a precursor of the stromelysin by culturing host cells transformed with a DNA sequence coding for the stromelysin precursor and cleaving the precursor to produce the stromelysin.
- 10 3. An essentially pure mammalian stromelysin, prostromelysin or preprostromelysin characterised in that the mammalian stromelysin, prostromelysin or preprostromelysin is human stromelysin, prostromelysin or preprostromelysin.
- 4 A mammalian stromelysin or prostromelysin produced by a process according to Claim 1.
- 15 5. A fusion protein comprising a heterologous protein and a mammalian stromelysin or prostromelysin.
6. A DNA sequence coding for the amino acid sequence of a mammalian stromelysin or prostromelysin or a precursor thereof provided that when the DNA sequence codes for rat stromelysin the  
20 DNA sequence is not the pTR1 cDNA sequence.
7. An expression vector including a DNA sequence coding for a mammalian stromelysin, prostromelysin or precursor thereof.
8. Host cells transformed with a vector according to Claim 7.
9. A pharmaceutical composition comprising a mammalian stromelysin  
25 and a pharmaceutically acceptable excipient.
10. A method of therapy comprising treating a patient with an effective amount of a mammalian stromelysin.



2/7

Fig. 3



( ) = other sites for these enzymes  
are on map but not shown

X = XbaI	N = NcoI
M = MboII	P = PvuII
B = BclI	A = AvaI
H = HindIII	S = Sau3A

3/7

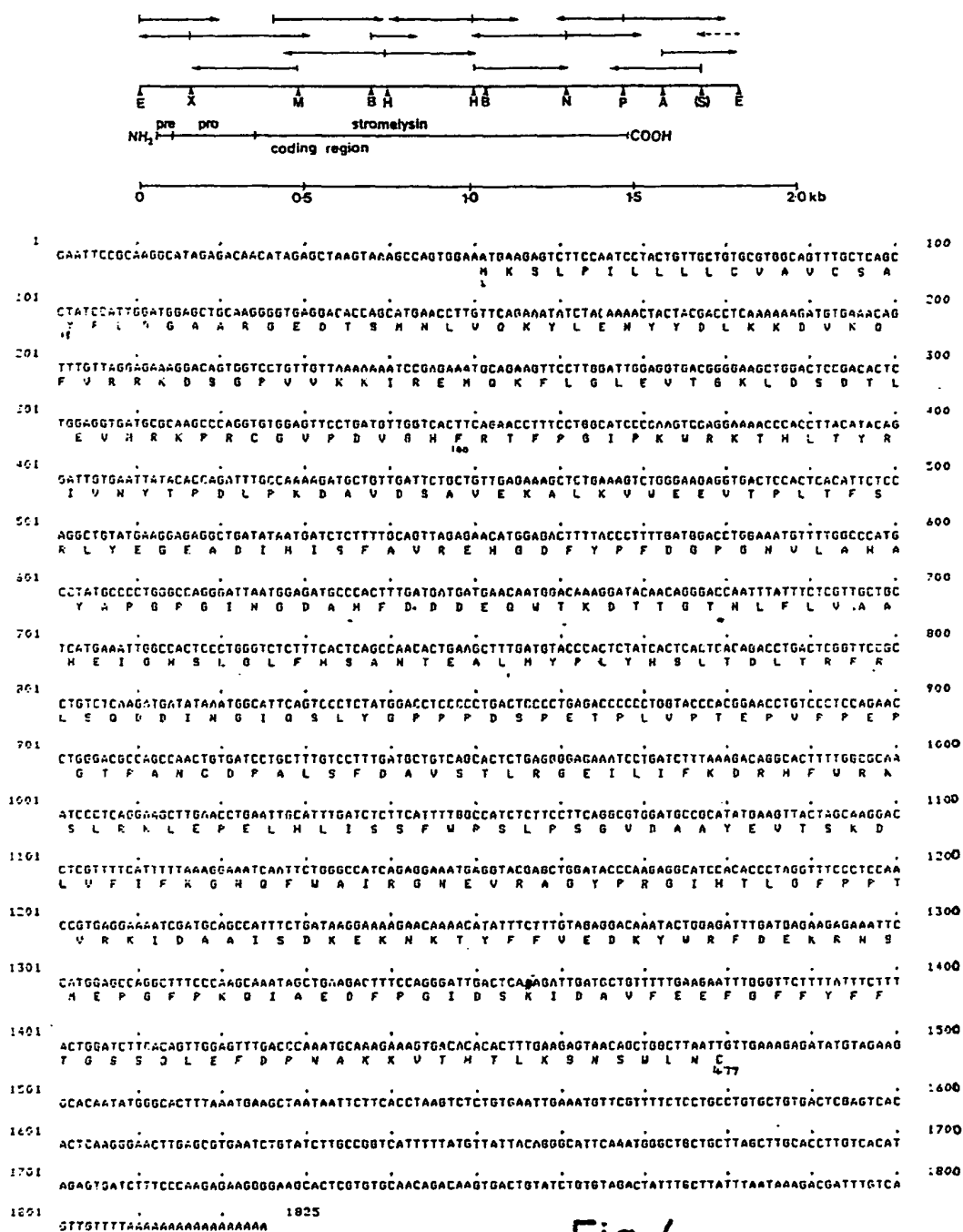


Fig. 4

SUBSTITUTE SHEET

4/7

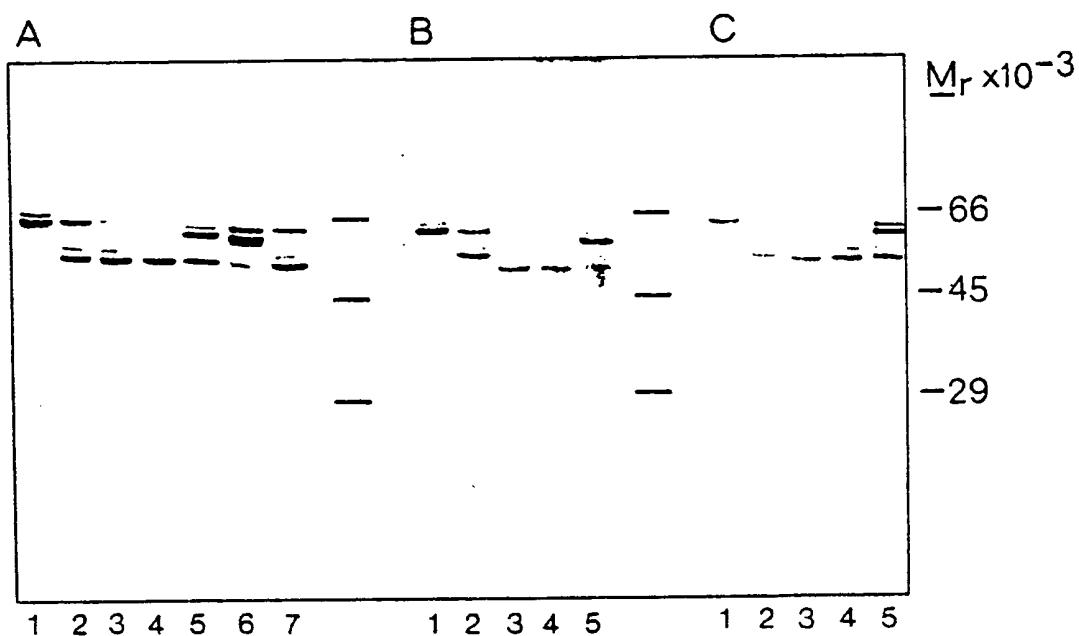


Fig. 5

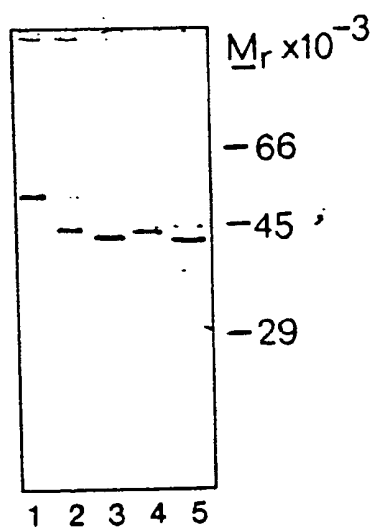
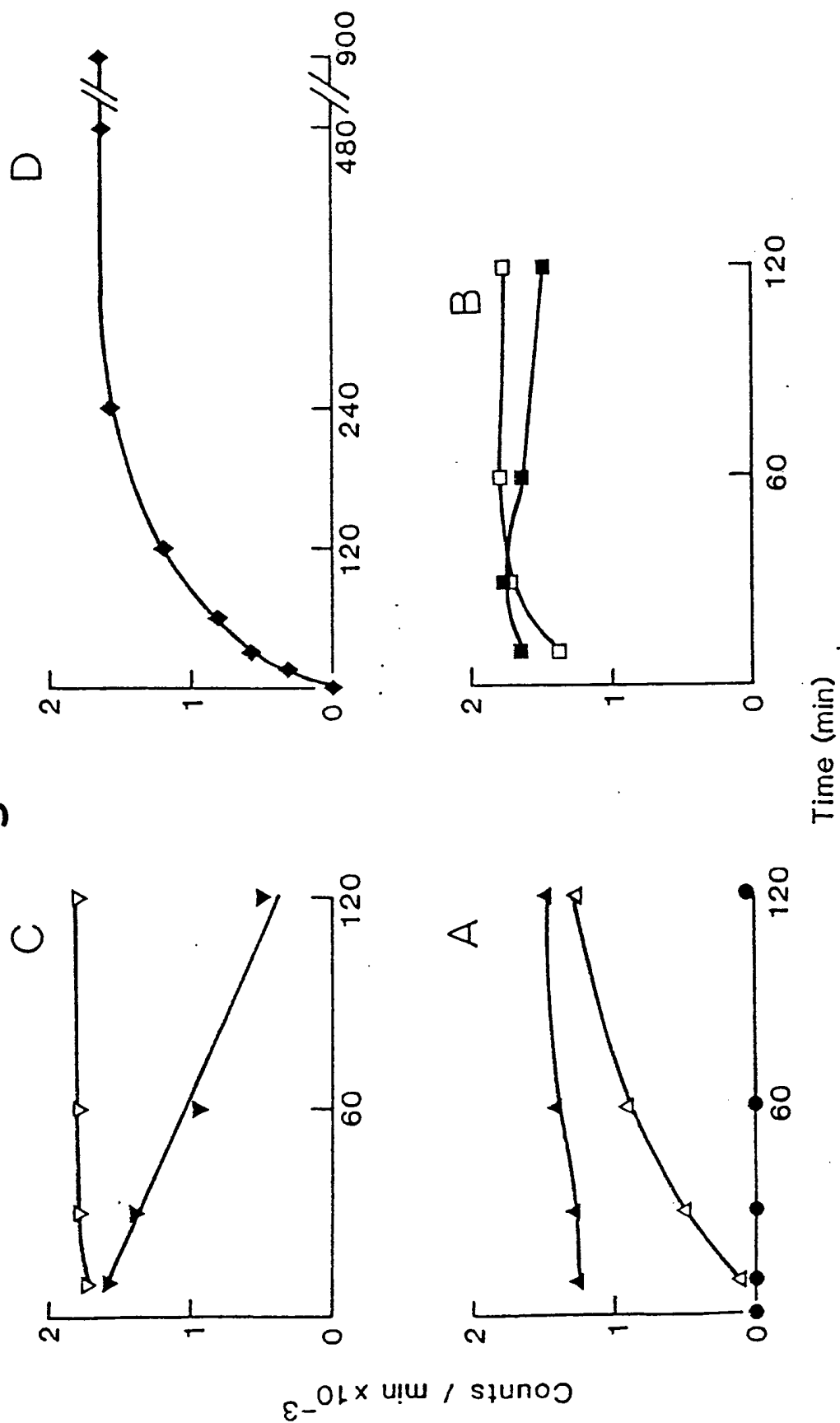


Fig. 9

SUBSTITUTE SHEET

5/7

Fig. 6





6/7

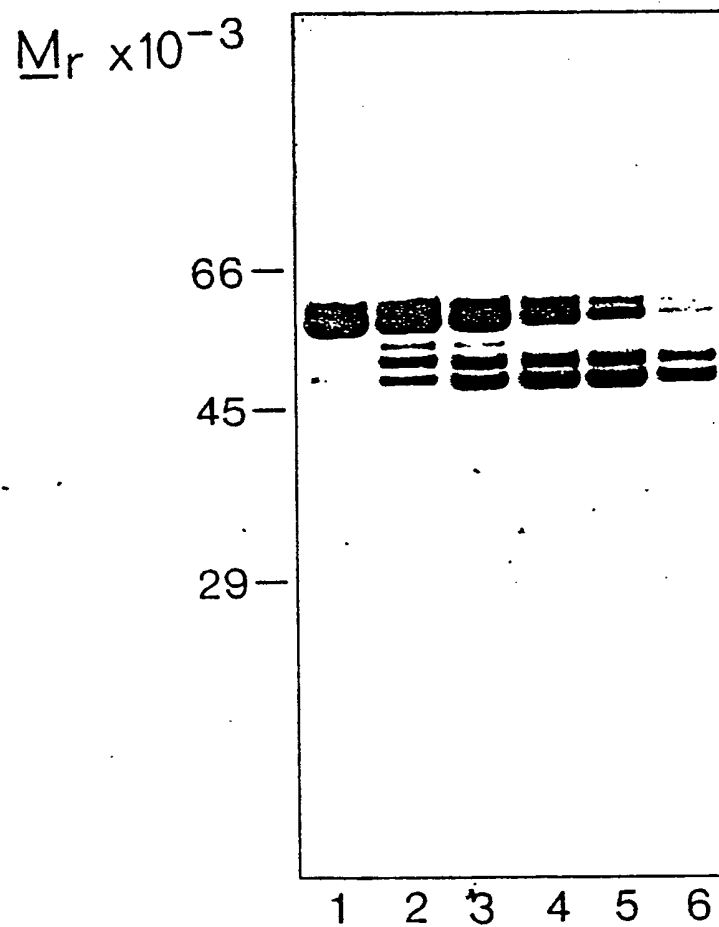


Fig. 7

SUBSTITUTE SHEET

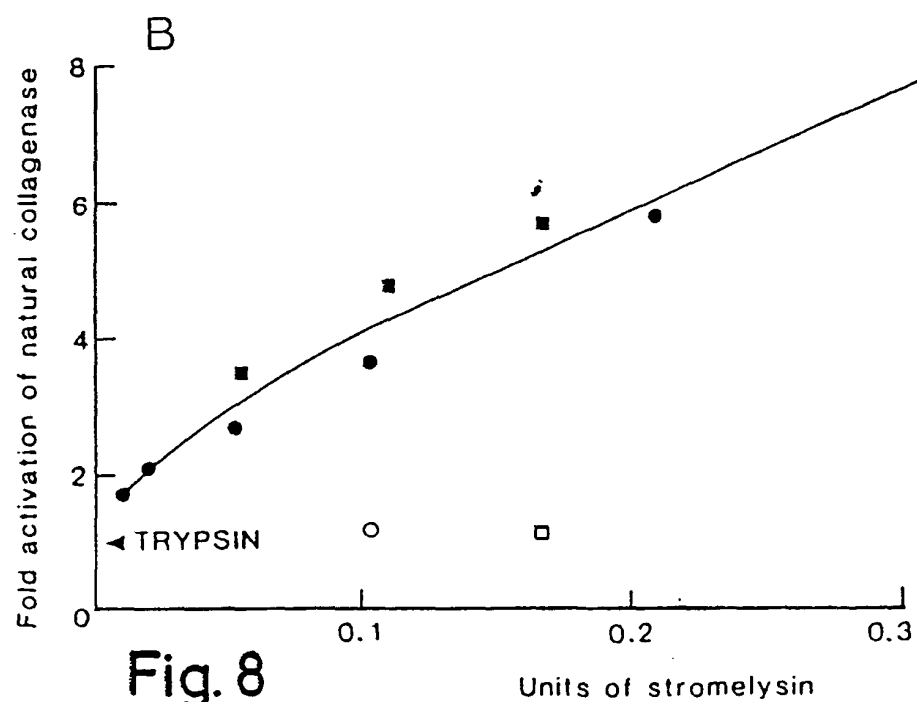
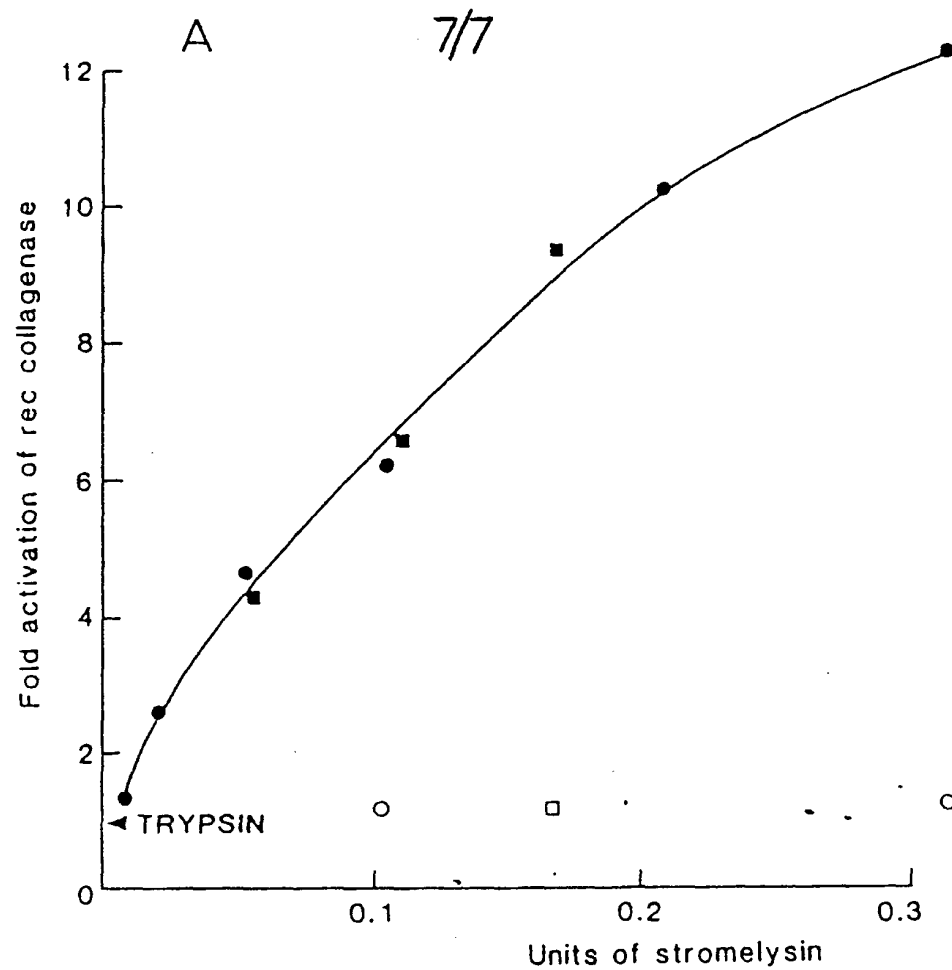



Fig. 8

SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00420

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC <sup>4</sup> : C 12 N 15/00; C 12 N 9/64; A 61 K 37/54; C 12 N-1/00		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
IPC <sup>4</sup>	C 12 N A 61 K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	The Journal of Biological Chemistry, volume 260, no. 22, 5 October 1985, The American Society of Biological Chemists, Inc., (US), J.R. Chin et al.: "Stromelysin, a connective tissue-degrading metallo- endopeptidase secreted by stimulated rabbit synovial fibroblasts in parallel with collagenase", pages 12367-12376 see the whole document	3,4 1,2,6-8
Y	--	
X	Biochem. J., volume 209, 1983, The Bio- chemical Society, (GB), W.A. Galloway et al.: "Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective- tissue components", pages 741-752 see the whole document	3,4 1,2,6-8
Y	cited in the application	
	--	
	./.	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
6th October 1987		19 NOV 1987
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		M. VAN MOL 

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	The EMBO Journal, volume 4, no. 6, 1985, IRL Press Limited, (Oxford, GB), L.M. Matrisian et al.: "Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts", pages 1435- 1440 see the whole document cited in the application	1,2,6-8
X	J. Cell Biol., volume 97, no. 2, part 5, 1983, S.M. Frisch et al.: "Molecular cloning of a cDNA encoding a secreted connective tissue-degrading metallo- proteinase induced by changes in cytoskeletal structure", page 460a, abstract 1740 see the whole abstract cited in the application	6 1,2,7,8
P,X	Biochem. J., volume 240, 1986, (GB), S.E. Whitham et al.: "Comparison of human stromelysin and collagenase by cloning and sequence analysis", pages 913-916 see the whole document	6
Y	EP, A, 0157604 (SANKYO CO., LTD) 9 October 1985 see the whole document	1,2,6-8
P,X	Journal of Cell Biology, volume 103, no. 5, part 2, 1986, (US), E.J. Clark et al.: "Synthesis and secretion of collagenase and stromelysin in SV40 transformed human ataxia-telangiectasia fibroblasts", page 257a, abstract 961 see the whole abstract	6
A	Chemical Abstracts, volume 105, no. 17, 27 October 1986, (Columbus, Ohio, US), S.M. Frisch: "Molecular biology of the stromelysin and collagenase genes", see page 316, abstract 148779t, & Diss. Abstr. Int. B 1986, 46(5), 1544	
A	Chemical Abstracts, volume 105, no. 3, 21 July 1986, (Columbus, Ohio, US), G.I. Goldberg et al.: "Human fibro- blast collagenase. Complete primary structure and homology to an oncogene transformation-induced rat protein", ./. .	

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

	see page 209, abstract 19690h, & J. Biol. Chem. 1986, 261(14), 6600-5	
P,A	GB, A, 2182665 (MONSANTO CO.) 20 May 1987	9,10
A	EP, A, 0115974 (INSTITUT PASTEUR) 15 August 1984 see the whole document	
A	EP, A, 0159497 (K.K. YAKULT HONSHA) 30 October 1985 see the whole document	9,10

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers ..... 10... because they relate to subject matter not required to be searched by this Authority, namely:

See PCT rule 39.1 (IV)      Methods for treatment of the human or animal  
body by surgery or therapy, as well as  
diagnostic methods.

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00420 (SA 17576)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/10/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0157604	09/10/85	JP-A- 60207583	19/10/85
GB-A- 2182665	20/05/87	None	
EP-A- 0115974	15/08/84	WO-A- 8402653	19/07/84
		FR-A- 2538702	06/07/84
		AU-A- 2410584	02/08/84
		JP-T- 60500413	28/03/85
		FR-A,B 2538701	06/07/84
EP-A- 0159497	30/10/85	JP-A- 60188066	25/09/85
		US-A- 4624924	25/11/86

For more details about this annex :  
see Official Journal of the European Patent Office, No. 12/82